EXHIBIT E

Proliferative But Not Nonproliferative Responses to Granulocyte Colony-Stimulating Factor Are Associated With Rapid Activation of the p21^{ras}/MAP Kinase Signalling Pathway

By Asad Bashey, Lyn Healy, and Christopher J. Marshall

Granulocyte colony-stimulating factor (G-CSF) can elicit responses that include proliferation, granulocytic differentiation, and activation of cellular functions in target cells. The biochemical pathways responsible for transduction of these signals from the G-CSF receptor (G-CSFR) have not been defined. In this report, we show that, in murine (NFS-60) and human (OCI-AML 1) myeloid leukemia cell lines and in murine pro-B–lymphocytic cells, BAF/B03, transfected with the murine G-CSFR, proliferative responses to G-CSF are associated with rapid activation of p42 and p44 MAP kinases and p21^{ras}. Truncation of the cytoplasmic portion of the murine G-CSFR at residue 646 but not at residue 739 abolished G-CSF–induced stimulation of cellular proliferation as well as activation of MAP kinase and p21^{ras} in transfected BAF/B03 cells. G-CSF–induced granulocytic differentiation of the murine leukemic cell line 32DC13(G) occurred in the absence of detectable activation of p42 MAP kinase. Nonproliferative responses to G-CSF in the human promyelocytic cell line HL-60 and in human neutrophils were similarly associated with no MAP kinase activation. These results imply that differing cellular effects of G-CSF may be involve the recruitment of differing signal transduction pathways with the p21^{ras}/MAP kinase pathway being limited to proliferative responses.

GRANULOCYTE colony-stimulating factor (G-CSF) is a glycoprotein cytokine that acts on cells committed to the granulocytic lineage to produce several different responses. These include proliferation and differentiation of granulocyte progenitors[1,2] and augmentation of the effector functions of mature neutrophils.[3,4] It can also act synergistically with other cytokines.[5-7]

These effects are mediated by the binding of G-CSF to a single class of specific, cell surface glycoprotein receptor (G-CSFR) of Mr 100 to 150 kD.[8] Analysis of the polypeptide sequence of the human[9,10] and murine[11] G-CSFR has shown that they belong to the hematopoietin receptor superfamily (HRS),[12] which includes the receptors for the majority of other hematopoietic growth factors. However, unlike other members of this family, G-CSFR binds its ligand with high affinity as a homodimer.[13] As with other members of the HRS, the cytoplasmic portion of the G-CSFR contains no recognizable protein kinase domains. Although phosphorylation on tyrosine residues of several proteins has been shown to be a consequence of G-CSF activity,[14,15] the exact components responsible for signal transduction from this receptor have not been determined.

The cellular proto-oncogene p21^{ras} has been shown to be an important mediator of signal transduction in mammalian cells.[16] The switch of this molecule from the inactive GDP-bound state to the active GTP-bound state by the action of receptor tyrosine kinases (RTK)[17,18] results from the association of the adaptor protein Grb2[19] and the guanine nucleotide exchange factor Sos[20-22] with autophosphorylated tyrosine residues on the receptor. Stimulated exchange factor activity then activates p21^{ras} by inducing the release of bound GDP, which is replaced by cytoplasmic GTP. The activation of p21^{ras} is associated with the downstream activation of a protein kinase cascade that includes the serine/threonine kinase Raf-1,[23] the dual specificity tyrosine-threonine kinase MAP kinase kinase (MAPKK),[24] and the 40- to 50-kD family of serine threonine kinases—the MAP kinases (p42^{mapk}, ERK2; p44^{mapk}, ERK1).[23,25]

A number of growth factors with hetero-oligomeric receptors that belong to the hematopoietin receptor superfamily, eg, interleukin-2 (IL-2), IL-3, IL-5, and granulocyte-macrophage–CSF (GM-CSF), have been shown to activate p21^{ras} [26,27] or the MAP kinases.[28-31] However, the role of this pathway in signalling from G-CSF remains unclear. Although GM-CSF produces a marked increase in MAP kinase activity in neutrophils,[29-31] Raines et al[30] found that G-CSF produced no major increase in MAP kinase activity in the same cells. Furthermore, the cytoplasmic domain of the murine G-CSFR is approximately 50% homologous to that of the IL-4 receptor[11] and both types of receptor appear to function as homodimers, transmission of a proliferative signal in response to IL-4 in the mast cell line MC-9 is apparently not associated with a significant increase in the p21^{ras}GTP/GDP bound ratio.[26,27]

In this study, we have examined whether signal transduction from the G-CSFR involves the p21^{ras}/MAP kinase pathway. We show that proliferative but not nonproliferative responses to G-CSF are associated with rapid activation of MAP kinase and/or p21^{ras}. By transfection of truncation mutants of the murine G-CSFR (mu-G-CSFR) into BAF3/B03 cells, the cytoplasmic domains of the receptor responsible for the activation of this pathway have been determined.

MATERIALS AND METHODS

Cell Culture

NFS-60 cells were provided by Dr J. Ihle (St Jude's Hospital, Memphis, TN), OCI-AML 1 cells were a gift from Dr E. McCulloch (Ontario Cancer Institute, Toronto, Ontario, Canada), and

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32DC13 cells were obtained from Dr J. Greenberger. NFS-60 cells,[32] 32DC13 cells,[33] and BAF/BO3 cells[34] were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3B conditioned medium (WCM)[35] as a source of mu-IL-3. HL-60 cells were grown in RPMI1640 with 10% FCS. OCI-AML1 cells[36] were cultured in α-modification of minimum essential medium (α-MEM) supplemented with 20% FCS and 10% 5637 conditioned medium. Human neutrophils were obtained from healthy volunteers and were purified by Ficoll-Hypaque density gradient centrifugation and dextran sedimentation.[37] They were confirmed by morphology to be more than 95% pure before use.

Cytokines

Recombinant human G-CSF (r-hu-G-CSF) was a generous gift of Amgen (Cambridge, UK). r-mu-GM-CSF and r-mu-IL-3 were purchased from Genzyme (Cambridge, MA).

Stimulation With Cytokines and Cell Lyses

For MAP kinase assays, exponentially growing cells (5×10^6 per point) were made quiescent by deprivation of growth factor (IL-3 for NFS-60, BAF-3, and 32DC13 cells; G-CSF for OCI-AML1; FCS for HL-60) for 6 to 18 hours depending on cell type. Human neutrophils were treated with 1 mmol/L diisopropylfluorophosphate before use. The cells were subsequently exposed to r-hu-G-CSF or r-mu-IL-3 or r-mu-GM-CSF as indicated. After incubation for the specified time at 37°C, the cells were lysed on ice in buffer containing 20 mmol/L Tris HCl (pH 8.0), 40 mmol/L Na pyrophosphate, 50 mmol/L NaF, 5 mmol/L $MgCl_2$, 100 μmol/L Na_3VO_4, 10 mmol/L EDTA, 1% (vol/vol) Triton X-100, 0.5 mmol/L (wt/vol) Na deoxycholate, 10 μg/mL aprotinin, 20 μg/mL leupeptin, and 3 mmol/L phenylmethylsulphonyl fluoride (PMSF) (reagents from Sigma, St Louis, MO). Cell debris and nuclei were removed by centrifugation at 16,000*g* for 10 minutes at 4°C. The supernate was stored at −70°C until used.

For analysis of $p21^{ras}$ bound GTP/GDP, exponentially growing cells (2×10^7 per point) were deprived of growth stimulus as above and metabolically labeled by incubation in phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing [^{32}P] orthophosphate 0.5 mCi/mL (US PBS.11, carrier free; Amersham, Amersham, UK) for 3 hours at 37°C. The cells were then stimulated with G-CSF as for the MAP kinase assays. Cell lysis, immunoprecipitation of $p21^{ras}$, and thin layer chromatography were then performed essentially as described by Downward et al,[38] except that Triton X114 was used as the lysing detergent and, after spinning out the nuclei, the lysates were incubated at 37°C for 2 minutes to separate the phases of the detergent. The detergent phase containing $p21^{ras}$ was then diluted 10-fold before immunoprecipitation with Y13-259 monoclonal antibody bound to protein G-sepharose (Sigma).

Assay of MAP Kinase Activation

Two polyclonal rabbit antisera (122 and 124)[25] generated against C-terminal MAP kinase peptides were used for this analysis. 122 is an immunoprecipitating antiserum that recognizes $p42^{mapk}$. Antiserum 124 recognizes both $p42^{mapk}$ and $p44^{mapk}$ on immunoblotting.

Immunoblot assay. Thirty to 50 μg of lysate protein boiled for 3 minutes in gel-loading buffer was loaded per well onto a 10% acrylamide, 0.16% bisacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, subjected to electrophoresis, and transferred electrophoretically onto a nitrocellulose filter (Schleicher & Schuell, Keene, NH). The filter was then blocked in TBST (20 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20) containing 5% nonfat dried milk for 1 hour at room temperature and probed with 124 for 1 hour at 1:15,000 dilution in blocking buffer. After washing in TBST, the filter was incubated with the secondary antibody (goat-antirabbit coupled to horseradish peroxidase; Pierce, Rockford, IL) and developed using the ECL detection system (Amersham). Activated (phosphorylated) MAP kinase appeared as a band of retarded mobility.

Immunoprecipitate MBP kinase assay (IP kinase assay). $p42^{mapk}$ was immunoprecipitated from 50 μg of cell lysate protein using 10 μL of 122 coupled to protein-A-sepharose (Sigma). After washing three times, the immunoprecipitates were suspended in 30 μL of kinase buffer (30 mmol/L Tris HCl, pH 8.0, 20 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$, 10 μmol/L ATP, 0.25 mg/mL myelin basic protein [MBP; Sigma], and 33 μCi/mL [γ^{32}P]ATP [5,000 Ci/mmol; Amersham]). The suspension was incubated with shaking at 30°C for 30 minutes. The reaction was terminated by the addition of 7.5 μL of 5× gel-loading buffer (1× = 62.5 mmol/L Tris HCl, pH 6.8, 2.3% wt/vol SDS, 5 mmol/L EDTA, 100 mmol/L dithiothreitol, 10% vol/vol glycerol). The mixture was heated at 100°C for 5 minutes and resolved on a 15% SDS-PAGE gel that was dried and autoradiographed.

Construction of mu-G-CSFR Deletion Mutants

mu-G-CSFR cDNA was a generous gift from S. Nagata (Osaka Biosciences Institute, Osaka, Japan). The cytoplasmic deletion mutants were constructed using the polymerase chain reaction (PCR) with a 5′ (sense) primer corresponding to nucleotides 1817-1838 containing a unique *Kpn* I site and 3′ (antisense) primers containing in frame termination codons after nucleotides 2195 (Δ 646) and 2481 (Δ 739), respectively. The deletion mutants and the full-length mu-G-CSFR were subcloned into pRcCMV (Invitrogen, San Diego, CA), which also contains a neomycin resistance gene under the control of the SV40 early promoter. All constructs were subject to nucleotide sequence analysis to confirm their identity.

Transfection Into BAF3/BO3 Cells and Isolation of Transfected Clones

The mu-G-CSFR and deletion mutants in pRcCMV were transfected into BAF/BO3 cells by electroporation in a BioRad gene pulser (BioRad, Hercules, CA). Cells (1×10^5) were transfected in 0.8 mL disposable cuvettes (BioRad) with 5 μg of plasmid at 250 V and 500 μF capacitance. Transfected cells were plated at low density (500/mL) in RPMI/10% FCS/10% WCM in 24-well plates. After 24 hours, geneticin (G418) was added at 2 mg/mL. Outgrowing clones were transferred to tissue culture flasks and grown continuously in G418.

Affinity Cross-Linking With ^{125}I G-CSF

^{125}I-labeled G-CSF was a generous gift from Amersham. Radiolabeled ligand (1 nmol/L) was incubated with 5×10^6 cells for 2 hours at 4°C and was then cross-linked with bis(sulfo-succinimidyl) suberate (BS^3; Pierce) as described by Larsen et al.[10]

Differentiation of 32DC13 Cells

Exponentially growing 32DC13 cells were washed free of WCM and incubated in RPMI/10% FCS with 50 U/mL of r-hu-G-CSF for 15 days. Aliquots were sampled on days 0, 5, 7, and 12 and were assessed for differentiation by morphology on May-Grünwald-Giemsa–stained slides, myeloperoxidase staining, and expression of GR-1 antigen.

Immunophenotyping and Assay of G-CSFR Using Flow Cytometry

Cells were labeled directly with a fluorescein (FITC)-conjugated antibody GR-1 (Pharmingen, San Diego, CA), a rat antibody di-

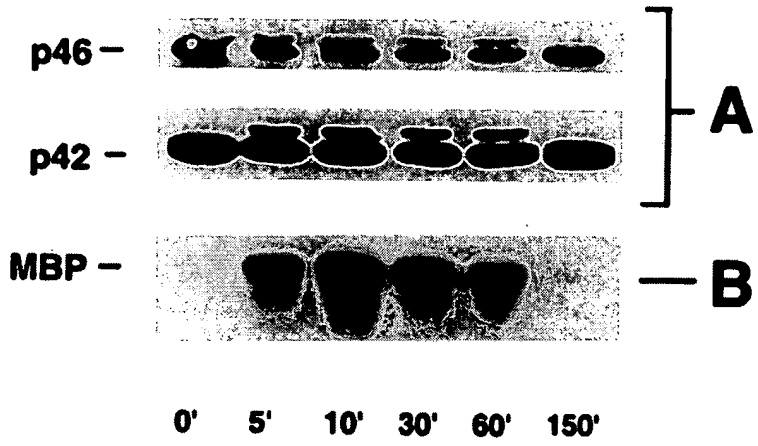

Fig 1. G-CSF rapidly activates MAP kinases in NFS-60 cells. IL-3–deprived NFS-60 were stimulated with r-G-CSF at 500 U/mL for 0 to 150 minutes as indicated. (A) Immunoblot assay. The activated forms of both p42 and p44(p44-46) MAP kinase are seen as bands of retarded mobility. (B) Immunoprecipitate MBP kinase assay.

rected against a mouse granulocyte-specific antigen expressed on granulocytes from all mouse strains.[39] Cell surface staining was performed using standard immunofluorescence techniques. An FITC-conjugated rat IgG_{2b} was included as a negative control.

The direct binding of phycoerythrin (PE)-conjugated G-CSF to its receptor was performed using the FCS50P Fluorokine G-CSF-PE flow cytometry system (R&D Systems, Minneapolis, MN). Briefly, 4×10^6 cells/mL were washed twice in a wash buffer (provided with the kit) to remove growth factors present in the culture medium and 10 μL of the G-CSF-PE reagent was added to 25 μL of cells and incubated on ice for 60 minutes. A control tube containing cells plus strepavidin-PE at the same concentration as the growth factor was also included. After incubation, cells were washed twice in wash buffer to remove unbound G-CSF-PE and the cells were resuspended in 200 μL phosphate-buffered saline before analysis.

Cells were analyzed using an FACScan (Becton Dickinson, Sunnyvale, CA) with an argon laser operating at 488 nm and 15 mW. The FITC filter had a 530/30 nm band pass and the PE filter a 585/42 nm band pass. Data were collected and analyzed using the LYSYS II package on a Hewlett-Packard 340 series.

Myeloperoxidase Staining

Cytospun cells were stained for myeloperoxidase using Sigma Kit 390A. The presence of intense brown-black intracellular granulation was indicative of neutrophils and their precursors.

RESULTS

Proliferative Responses to G-CSF Are Associated With Rapid Activation of MAP Kinases

NFS-60 is an IL-3–dependent murine myeloid leukemic cell line that proliferates in response to G-CSF in the absence of detectable differentiation or functional alterations.[32] When quiescent (IL-3 deprived for 4 to 6 hours) NFS-60 cells were exposed to r-G-CSF (500 U/mL), they showed a rapid increase in the $p42^{mapk}$ kinase activity as measured by the MBP kinase activity of antiserum 122 immunoprecipitates (Fig 1). This activation was detectable at 2 to 5 minutes after G-CSF addition and remained elevated at 60 minutes, but had diminished significantly at 150 minutes. This increased enzymatic activity correlated temporally with the appearance of slower migrating forms of both $p42^{mapk}$ and $p44^{mapk}$ on immunoblots of cell lysates probed with antiserum 124 (Fig 1). We have previously shown these decreased mobility forms of $p42^{mapk}$ and $p44^{mapk}$ to result from the phosphorylation of these enzymes on both threonine and tyrosine residues.[25] This phosphorylation is a prerequisite for the activation of the enzymatic function of these molecules.[40]

G-CSF–induced activation of MAP kinases in NFS-60 cells was dose-dependent (minimal concentration for stimulation, 10 to 50 U/mL), with a maximal level at 100 to 500 U/mL (data not shown). The kinetics and intensity of MAP kinase activation in NFS-60 cells was similar to the MAP kinase response to r-mu-IL-3 in these cells (data not shown) and to that seen in mammalian fibroblasts in response to growth factors binding receptor tyrosine kinases (RTKs), eg, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)[25] (unpublished results). The rapid stimulation of MAP kinases seen suggests that MAP kinase activation is a direct consequence of G-CSF binding to these cells rather than a result of the secretion of autocrine growth factors by these cells in response to G-CSF. Similar rapid activation of MAP kinases (maximum activity, 5 to 10 minutes) was observed in the human myeloid leukemia line OCI-AML1, which also demonstrates a proliferative response to G-CSF.[36] Although the latter cells responded by early activation of MAP kinase, they showed a more rapid decrease in of enzyme activity than NFS-60 cells with no detectable activated kinase at 30 to 60 minutes poststimulation.

G-CSF Stimulates a Rapid Increase in GTP Association With $p21^{ras}$ in NFS-60 Cells

Although MAP kinases have been shown to lie downstream of $p21^{ras}$ activation in tyrosine kinase signalling[23,41,42] and activation of $p21^{ras}$ appears to lead to activation of the MAP kinases, it is possible that MAP kinases may also be activated by $p21^{ras}$ independent alternative pathways.[43-45] To determine whether the rapid activation of MAP kinases on G-CSF–stimulated cell proliferation is associated with $p21^{ras}$ activation, we measured $p21^{ras}$ bound GTP/GDP ratios after exposure to G-CSF. As indicated in Fig 2, exposure of growth factor starved NFS-60 to G-CSF led to the rapid increase of $p21^{ras}$ in GTP bound form. Maximum activation of $p21^{ras}$ (58% GTP-bound) had occurred

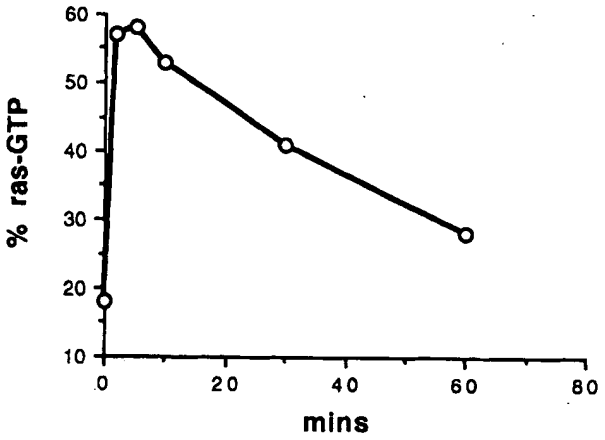

Fig 2. Increase in p21^{ras} bound GTP in response to G-CSF stimulation of NFS-60 cells. Growth factor-deprived NFS-60 cells were labeled with ^{32}P orthophosphate for 3 hours and then stimulated with r-G-CSF (500 U/mL). Guanine nucleotides bound to p21^{ras} were analyzed at the indicated times (minutes) after exposure to r-G-CSF. p21^{ras}/GTP is shown as a percentage of total p21^{ras} bound guanine nucleotide (GTP + GDP) determined with a Molecular Dynamics Phosphorimager.

by 5 minutes of exposure with a subsequent decline so that, at 60 minutes, only 28% of p21^{ras} was GTP bound. The kinetics of p21^{ras} activation were similar to that of MAP kinase activation, implying that, in these cells, the activation of MAP kinase results at least in part from an increase in cellular p21^{ras} bound GTP.

Deletion of the mu-G-CSFR at Amino Acid 646 (Δ646) But Not at Amino Acid 739 (Δ739) Abolishes G-CSF–Induced Activation of MAP Kinase and p21^{ras}

It has previously been shown that untransfected BAF/B03 cells do not express the G-CSFR and are unresponsive to G-CSF and that these cells become responsive by proliferation to G-CSF when stably transfected with either the human or murine G-CSFR.[46,47] To assess the role of MAP kinase and p21^{ras} activation in the G-CSF response of transfected cells, the full-length mu-G-CSFR and mutants deleted at residues 646 (Δ 646) and 739 (Δ739) (Fig 3) were subcloned into vector pRcCMV containing a neomycin resistance gene and electroporated into BAF/BO3 cells. Stably transfected clones were isolated by limiting dilution and the ability to grow in G418 (2 mg/mL). Expression of transfected mu-G-CSFR in the isolated clones was assessed by affinity cross-linking to ^{125}I-labeled hu-G-CSF (Fig 4). The transfected clones expressed G-CSF receptors of the appropriate molecular weight. Clones chosen for further analysis expressed similar levels of transfected receptor.

Parental BAF/B03 cells require IL-3 for survival and proliferation and cannot proliferate in response to G-CSF. However, BAF/B03 transfectants expressing the full-length mu-G-CSFR [t(812)] and those expressing Δ739 [t(Δ739)] could proliferate in G-CSF (500 U/mL) in the absence of IL-3 (Fig 5). Transfectants expressing Δ646 [t(Δ646)] or pRcCMV alone and parental BAF/B03 cells did not proliferate or incorporate tritiated thymidine in response to G-CSF and failed to remain viable in RPMI/10% FCS with r-G-CSF. If appropriately diluted, full-length and Δ734 transfectants could grow indefinitely in RPMI/10% FCS/r-G-CSF in the absence of IL-3 with no evidence of differentiation or functional alteration.

Activation of MAP kinase and p21^{ras} in response to G-CSF in the BAF-3 transfectants correlated with the ability of G-CSF to induce proliferation in these cells. Exposure of IL-3–starved t(812) or t(Δ739) to r-G-CSF was associated with rapid activation of both MAP kinase and p21^{ras} (Fig 6), whereas t(Δ646) parental BAF/B03 cells and transfectants expressing pRcCMV alone showed no detectable response. The kinetics of the MAP kinase and p21^{ras} response to G-CSF in t(812) and t(Δ739) was similar to that observed for G-CSF responses in NFS-60 cells. All BAF-3 transfectants and the parental cell line showed rapid activation of MAP kinase and p21^{ras} in response to the readdition of IL-3 to cells starved of this growth factor (data not shown).

MAP Kinases Are Not Activated in the Transduction of Nonproliferative Signals From the G-CSFR

To assess whether MAP kinase activation is a universal component of G-CSFR signal transduction, activation of these enzymes was assessed in cell types that show nonproliferative responses to G-CSF. Human peripheral blood neutrophils are terminally differentiated cells that express the G-CSFR[48] and respond to G-CSF with augmentation of effector functions.[4,49] Exposure of freshly isolated peripheral blood neutrophils to r-G-CSF was associated with no detectable activation of MAP kinase on the immunoblot assay (Fig 7), whereas exposure to physiologic concentrations of GM-CSF produced a rapidly detectable activation.

To study the role of MAP kinase activation in G-CSF–induced granulocytic differentiation, a variant of the murine myeloid leukemic cell line 32DC13(G) was used. This variant showed no detectable proliferation as estimated by cell counting or tritiated thymidine incorporation when ex-

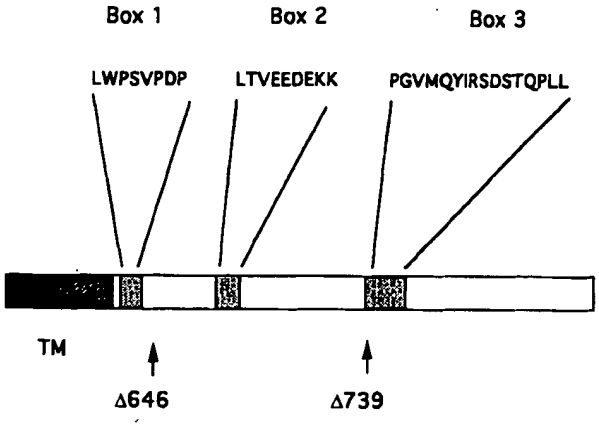

Fig 3. Schematic representation of the transmembrane (shaded) and cytoplasmic (open) regions of the murine G-CSFR. The termination points of the deletion mutants are indicated. The amino acid sequences and positions of the three boxes conserved between different members of the hematopoietin receptor family are shown. TM, transmembrane region.

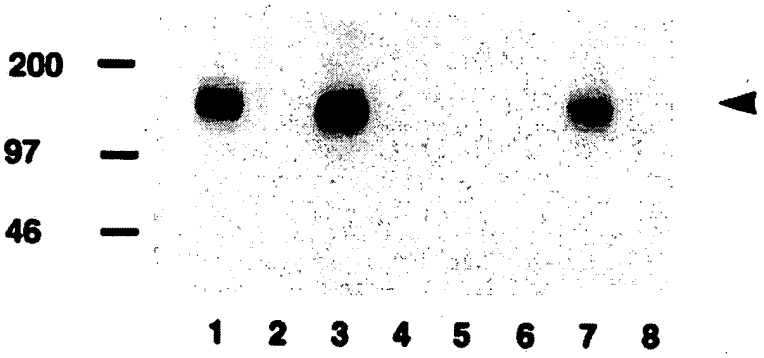

Fig 4. Expression of the mu-G-CSFR by BAF/B03 transfectants. Cells were incubated with ^{125}I G-CSF in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 200-fold excess of unlabeled G-CSF and cross-linked with BS^3. Lanes 1 and 2, t(812); lanes 3 and 4, t(Δ739); lanes 5 and 6, parental BAF/B03 cells; lanes 7 and 8, NFS-60 cells. The cross-linked murine G-CSFR is indicated by an arrow. The positions of the molecular weight markers used are shown on the left.

posed to a wide range of concentrations of G-CSF in the absence of IL-3 (data not shown). However, G-CSF treatment (>20 U/mL) in the absence of IL-3 was associated with progressive granulocytic differentiation of these cells with almost complete transformation to neutrophil-like cells by day 12 when assessed by cell morphology (neutrophils: 0% on day 0, 62% on day 7, 89% on day 11), expression of myeloperoxidase (0% on day 0, 92% on day 11), or the murine neutrophil-specific antigen GR-1 (Fig 8). Analysis of MAP kinase immunoblots made from these cells on G-CSF stimulation showed no detectable activation (Fig 7), although reexposure of the cells to IL-3 produced a rapid MAP kinase response.

G-CSF also induces the human promyelocytic cell line HL-60 to differentiate into granulocytes. This effect was not seen to be associated with significant MAP kinase activation (Fig 7), although r-hu-GM-CSF at 10 ng/mL produced strong activation of p42^{mapk} in these cells within 10 minutes. In all three cell types showing nonproliferative responses to G-CSF, immunoblotting showed similar levels of p42^{mapk} and p44^{mapk} to those in cells with proliferative responses. Furthermore, no activation of MAP kinases was seen with concentrations of r-G-CSF up to 50,000 U/mL or periods of exposure up to 6 hours (data not shown).

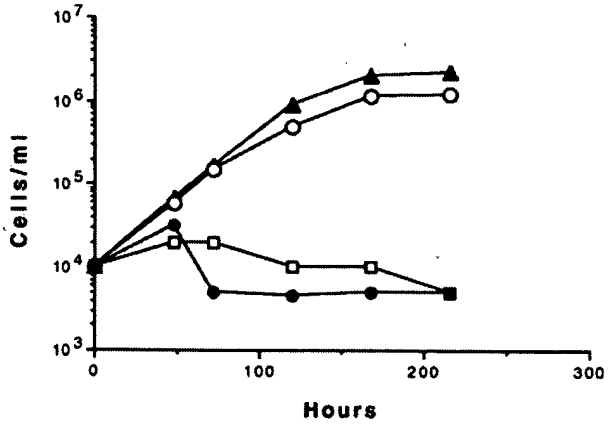

Fig 5. Transfectants t(812) and t(Δ739) but not t(Δ646) proliferate in response to G-CSF. Transfectants were washed in RPMI alone and then incubated (10^4/mL) in RPMI/10% FCS with G-CSF (500 U/mL) and aliquots were counted on subsequent days. (□) Parental BAF/B03 cells; (○) t(812); (▲) t(Δ739); (●) t(Δ649).

DISCUSSION

p21^{ras} and the MAP kinases are components of a signalling pathway that regulates proliferation and differentiation of eucaryotic cells by receptor tyrosine kinases.[50] Recent investigations have defined a number of the components of this pathway between the RTKs and the MAP kinases.[51] This pathway has also been shown to be involved in signalling from the HRS, but the specific biochemical link between these receptors and the activation of p21^{ras} and MAP kinases is less well defined. Although G-CSFR belongs to the HRS, it is unusual within this group in functioning as a homodimer.[13] Furthermore, its cytoplasmic portion which is required for signal transduction is homologous to the IL-4R, which can apparently induce cell proliferation without detectable activation of p21^{ras} or MAP kinases.[26-28] In this study, we have used cell types showing differing responses to G-CSF to assess the involvement of the p21^{ras} and MAP kinase activation in signal transduction from the G-CSFR.

We show that, in cells that proliferate in response to G-CSF [NFS-60, OCI-AML1, BAF3/B03 transfectants t(812 and Δ739)], exposure to G-CSF is associated with a rapid activation of MAP kinase (peak activity, 5 to 10 minutes). The speed of this response implies that MAP kinase activation is a direct consequence of receptor-ligand binding in these cells rather than the result of the secretion of other growth factors with autocrine activity. In NFS-60 cells and t(812 and Δ739) exposure to G-CSF also led to a rapid increase in cellular p21^{ras} bound GTP. The kinetics of p21^{ras} and MAP kinase activation were similar. By analogy, with other systems,[50] it is likely that in these cells p21^{ras} activation is at least partly responsible for the MAP kinase activation observed with G-CSF. Increase in cytoplasmic p21^{ras}-GTP has been shown to lead to downstream activation of the MAP kinases,[23,25,41] which occurs through a cascade of phosphorylation involving the serine-threonine kinase Raf-1 and the tyrosine/threonine kinase MAP kinase kinase (MAPKK).[51] The physical association of p21^{ras}-GTP and Raf-1 has recently been shown,[52-56] implying direct activation or activation through a multimeric complex. The use of the interfering dominant negative mutant of p21^{ras} (S17N-Ras)[57] with asparagine substituted for the serine at position 17 has shown that MAP kinase activation in response to

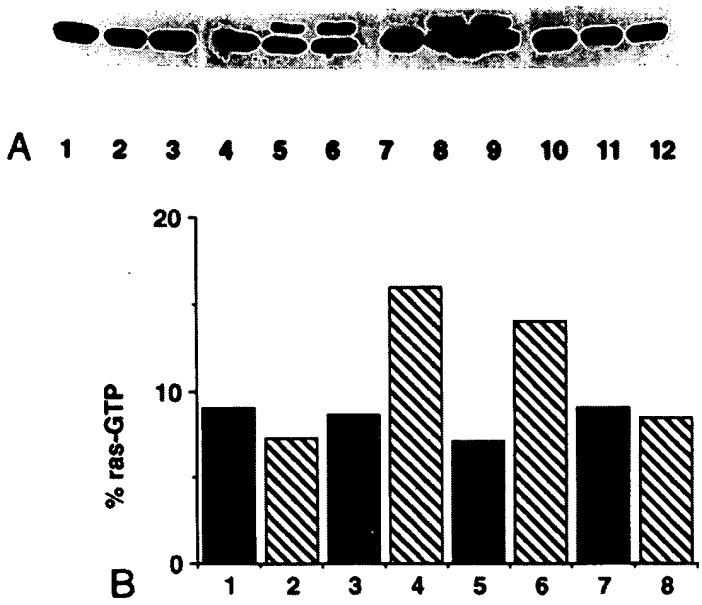

Fig 6. G-CSF activates MAP kinase and p21^{ras} in BAF/B03 transfectants t(812) and t(Δ739), but not in t(Δ646) or parental BAF/B03 cells. (A) p42^{mapk} activation. Immunoblot assay of IL-3–starved parental BAF/B03 cells (lanes 1 through 3), t(812) (lanes 4 through 6), t(Δ739) (lanes 7 through 9), and t(Δ646) (lanes 10 through 12) stimulated with r-G-CSF (500 U/mL) for 0 minutes (lanes 1, 4, 7, and 10), 5 minutes (lanes 2, 5, 8, and 11), or 10 minutes (lanes 3, 6, 9, and 12). (B) p21^{ras}/GTP as a percentage of total cellular p21^{ras} bound to GTP or GDP before (columns 1, 3, 5, and 7) or after (columns 2, 4, 6, and 8) stimulation with 500 U/mL G-CSF for 5 minutes. Parental BAF/B03 cells, columns 1 and 2; t(812), columns 3 and 4; t(Δ739), columns 5 and 6; t(Δ646), columns 7 and 8.

certain stimuli, eg, phorbol ester, can occur at least partly through a *ras*-independent pathway.[42,43] Whether such mechanisms are also involved in G-CSF–induced MAP kinase activation is currently undetermined because we have found it difficult to express S17N-Ras in hematopoietic cells.

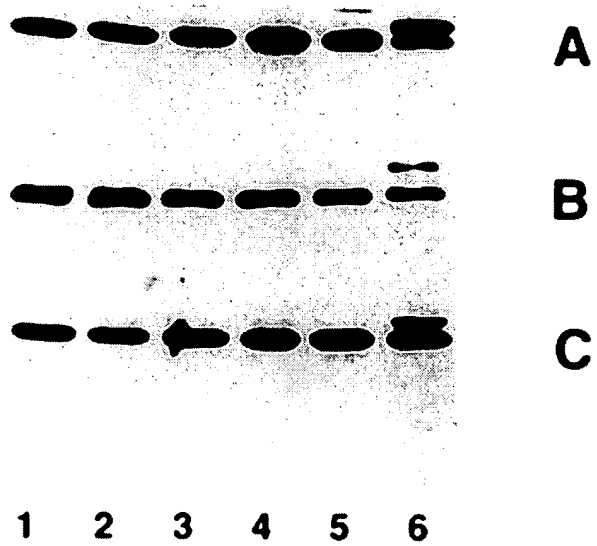

Fig 7. Nonproliferative responses to G-CSF in human neutrophils, HL-60 cells, and 32DC13(G) cells are not associated with activation of p42^{mapk}. Immunoblot assay of p42^{mapk} from lysates of unstimulated cells (lane 1), and cells stimulated for 5 minutes (lane 2), 10 minutes (lane 3), 30 minutes (lane 4), and 60 minutes (lane 5) with 500 U/mL of r-G-CSF. (A) Human neutrophils. Lane 6, stimulation with r-hu-GM-CSF 50 ng/mL for 10 minutes. (B) 32DC13(G) cells. Lane 6, stimulation with r-mu-IL-3 50 U/mL for 10 minutes. (C) HL-60 cells. Lane 6, stimulation with r-hu-GM-CSF 50 ng/mL for 10 minutes. The activated form of p42^{mapk} is seen as a band of retarded mobility.

In cell types that express the G-CSFR but show nonproliferative responses to G-CSF, MAP kinase activation by G-CSF was either undetectable or very weak, although other ligands, eg, IL-3 and GM-CSF, could activate MAP kinase. In a subclone of the cell line 32DC13(G), G-CSF induced almost complete granulocytic differentiation without detectable proliferation. No p42^{mapk} mobility shift could be demonstrated in response to G-CSF in these cells. The findings for human neutrophils are similar to those of Raines et al,[30] who found that stimulation with G-CSF produced only a minimal enzymatic activation of MAP kinase, whereas GM-CSF induced a 7- to 10-fold increase in activity. This observed disparity between GM-CSF and G-CSF in ability to induce MAP kinase activation in neutrophils is seen despite the considerable overlap in the functional response of these cells to the two ligands. Both GM-CSF and G-CSF increase the survival of granulocytes and enhance chemotaxis, phagocytosis, antibody-dependent cellular toxicity, and priming of the respiratory burst produced by the chemotactic peptide FMLP,[49,58-60] although GM-CSF additionally stimulates direct triggering of superoxide release and adhesion to cultured endothelium, which are not produced by G-CSF.[3,61] Whether activation of the p21^{ras}/MAP kinase pathway is essential in transducing the signal for these differential responses remains to be determined.

Although normal granulocytic progenitors can be demonstrated to proliferate in response to G-CSF, this ability to proliferate is lost as cells progress down the granulocytic lineage, with later responses to G-CSF being that of enhanced differentiation and the functional activation.[13] Uncoupling of the activated receptor from the p21^{ras} and MAP kinase activation could be a means of achieving the loss in proliferative capacity to G-CSF seen in granulocytic progenitors progressing through normal hematopoiesis. The components linking the G-CSFR to the p21^{ras}/MAP kinase path-

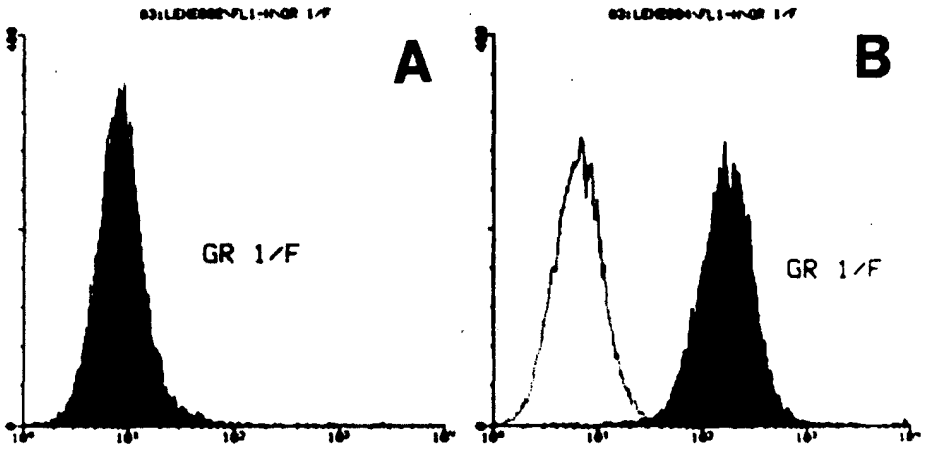

Fig 8. Differentiation of 32DC13(G) cells in response to G-CSF. FACS analysis of expression of granulocytic antigen GR-1 (see Materials and Methods) (X-axis): before (A) and 11 days after (B) exposure to r-G-CSF (300 U/mL).

way are not clearly defined. For the β-chain of the IL-2R that shows some homology to the G-CSFR in its cytoplasmic domain, recruitment of the $p21^{ras}$/MAP kinase pathway depends on association of the activated receptor with the nonreceptor tyrosine kinases (NRTKs) $p56^{lck}$,[62,63] $p59^{fyn}$, or $p53/56^{lyn}$[64,65] with subsequent enhancement of their tyrosine kinase activity. If an NRTK is also involved in linking the G-CSFR to this pathway, then decreased levels of expression of this NRTK or inactivation by other means may effect uncoupling of the receptor from pathway and from cellular proliferation.

BAF/BO3 cells stably transfected with full-length murine G-CSFR t(812) or with the truncation mutant t(Δ739) but not those transfected with t(Δ646) or vector alone acquired the ability to proliferate indefinitely in the presence of G-CSF. These results are in agreement with those of Fukunaga et al,[46] who showed that a proliferative response could be obtained with murine G-CSFR truncated at residue 725 but not at residue 654. Similarly, it has recently been shown that the human G-CSFR truncated at residue 688 can still induce BAF/B03 proliferation in response to G-CSF, but that truncated at residue 657 could not.[66] In each case the data suggests that two boxes (boxes 1 and 2) of homology between G-CSFR and other hematopoietin receptors (located at residues 630-643 and 674-683 of the murine G-CSFR)[46] must be retained for the transmission of a proliferative signal to occur. We have shown that BAF/B03 transfectants [t(812) and t(Δ739)] that were capable of proliferation in response to G-CSF also show rapid activation of MAP kinase and $p21^{ras}$ on exposure to this ligand, whereas t(Δ646) and control BAF3/B03 cells showed no detectable $p21^{ras}$ and MAP kinase activation and could not proliferate in G-CSF–containing medium. However, it remains to be determined whether the region of the G-CSFR responsible for proliferation induction corresponds exactly with that responsible for the activation of $p21^{ras}$/MAP kinase pathway. It has been shown that for the β-chain of the IL-2R transfected into BAF3/B03 cells, activation of $p21^{ras}$ and subsequent induction of c-*fos* and c-*jun* depends on a domain of the receptor that is also responsible for association of the activated receptor with NRTKs.[67] This domain is known as the acidic region and lies in a C-terminal position to the two boxes of cytoplasmic homology (boxes 1 and 2) between hematopoietin receptors. Deletion of this region abolishes c-*fos*/c-*jun* induction by IL-2, but does not abrogate IL-2–induced proliferation. Deletion of a region of the human G-CSFR (residues 687-721) (equivalent to residues 686-720 of the murine G-CSFR) that is similarly placed with respect to conserved boxes 1 and 2 as the acidic region of the IL-2R, although showing only limited homology to it, leads to a suboptimal proliferative response to G-CSF in BAF3/B03 cells and completely abolishes the G-CSF–induced expression of acute-phase proteins in transfected hepatoma cells.[66]

Among the hematopoietin receptor family, G-CSFR is most closely related to gp130,[46] which acts as the signalling chain for the IL-6R, the leukemia inhibitory factor receptor (LIFR) and the receptor for ciliary neurotrophic factor (CNTF).[68] In addition to boxes 1 and 2, G-CSFR and gp130 show a third homologous box (conserved box 3) (amino acids 68% similar) located in a more C-terminal position (residues 738-753 of mu-G-CSFR)[46] (Fig 3). However, we have shown that deletion of this region of the G-CSFR impairs neither the proliferative response to G-CSF nor the associated activation of the $p21^{ras}$/MAP kinase pathway (Fig 6). Like G-CSF, ligands that signal through gp130 are capable of inducing certain target cells to differentiate.[69-71] Furthermore our data suggests that, for G-CSF, this may involve signalling through a pathway not involving activation of $p21^{ras}$ or MAP kinase. One possibility to be investigated in the future is that the C-terminal box is specifically involved in generation of nonproliferative signals from these receptors.

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